View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Estudo Geral

Accred Qual Assur (2008) 13:299–304
DOI 10.1007/s00769-008-0374-x

PRACTITIONER'S REPORT

Production of "in house" reference materials for ELISA screening of bovine urine and liver samples for clenbuterol

Isabel Pinheiro · Bruno Jesuíno · Jorge Barbosa · Fernando Ramos · José Matos · Maria Irene Noronha da Silveira

Received: 24 September 2007 / Accepted: 1 February 2008 / Published online: 4 March 2008
© Springer-Verlag 2008

Abstract Clenbuterol screening of bovines is done by analysis of urine, for monitoring living animals, and liver, for monitoring animals after slaughter. ELISA has generally been used as the main method for these purposes. Nevertheless, in Europe, methods must be validated according to Commission Decision (EC) 657/2007 criteria, i.e. by use of reference materials. Production of "in house" reference materials is a possibility, but the homogeneity, storage temperature, and period of stability of these materials must be investigated in the laboratory itself. This paper reports GC–MS evaluation of an "in-house"-produced batch of aliquots of bovine urine and liver, fortified with 10.0 ng/ml and 10.0 ng/g clenbuterol, respectively, and stored at −20 °C and at −60 °C. For urine stored for 20 weeks at −20 °C and at 60 °C the stability of clenbuterol was proved at the 95% confidence level. For liver, however, it was demonstrated at the same confidence level that clenbuterol was highly unstable during storage for 20 weeks at either of the temperatures studied.

Keywords Clenbuterol · Reference materials · Stability · ELISA · GC–MS · Urine · Liver · "In house"

I. Pinheiro
Laboratório Regional de Veterinária,
Direcção de Serviços de Veterinária,
Direcção Regional do Desenvolvimento Agrário,
Vinha Brava, 9700-236 Angra do Heroismo, Portugal

B. Jesuíno · J. Barbosa
Laboratório Nacional de Investigação Veterinária,
Estrada de Benfica, 701-1549-011 Lisboa, Portugal

F. Ramos (✉) · M. I. N. da Silveira
Grupo de Bromatologia, Centro de Estudos Farmacêuticos,
Faculdade de Farmácia da Universidade de Coimbra,
3000-295 Coimbra, Portugal
e-mail: fjramos@ci.uc.pt

J. Matos
Departamento de Ciências Agrárias, Centro de Biotecnologia dos Açores, Universidade dos Açores, Terra-Chã,
9701-851 Angra do Heroismo, Portugal

Introduction

The use of clenbuterol and other beta-agonists as growth promoters in animal production is an illegal practice throughout the EU [1, 2]. However, there have been some reports of clenbuterol food contamination in several European countries, including Spain [3, 4], France [5], Italy [6–8], Portugal [9] and in China [10].

In order to implement measures to monitor the prohibition, legislative acts have been issued in the EU that have led to the elaboration of residue-control plans for the collection of samples and their laboratory analysis in all EU Member States [1]. Bovine urine and liver were selected as matrices for monitoring living and slaughtered animals, respectively.

Within this context, residue analysis laboratories generally follow a strategy that comprises two distinctive steps—a first, screening, step, in which a large number of samples are analysed with a method that guarantees a minimum of false negatives, and a second, confirmation, step in which samples that were positive in screening tests are analysed by a method that makes it possible to identify the molecular structure of the suspected substance. Both screening and confirmatory methods of clenbuterol evaluation must be validated according to procedures and performance criteria that are common to all European laboratories approved for official monitoring of residues, to guarantee the quality and comparability of the analytical results [1]. Method validation should be achieved by use of

certified reference materials or, in their absence, by use of blank samples spiked with a known concentration of the substance under analysis, in order to determine the necessary performance data, for example the method's accuracy. Until now, for analysis of clenbuterol in bovine urine and liver, only the certified reference materials (CRM), approved by the Community Bureau of Reference (BCR), were available. These contain two beta-agonists (clenbuterol and salbutamol) for urine [11], and three beta-agonists (clenbuterol, salbutamol and terbutaline) for liver [12].

ELISA (enzyme-linked immunosorbent assay) screening tests for clenbuterol are capable of detecting more than one beta-agonist simultaneously and cannot be validated using these CRM, because ELISA methods give a result for the total concentration of the various beta-agonists, each of which has its own specific cross-reaction and recovery. For this reason blank samples fortified with clenbuterol at different concentrations are used in multidetection beta-agonist screening tests in order to monitor the performance of the method. However, using these spiked blank samples for analysis does not enable deduction of whether the observed variation is due solely to a particular day's recovery or whether it is also caused by random or systematic errors in the preparation of the spiked samples. This doubt prevents trustworthy determination of the mentioned performance data, which is mandatory for method validation. Thus, the objective of this study was to evaluate, by GC–MS, the possibility of producing "in house" reference materials for ELISA screening, consisting of urine and liver aliquots exogenously fortified with clenbuterol.

Materials and methods

Reagents

Clenbuterol was purchased from Sigma (St Louis, MO, USA). The internal standard (IS) used in GC–MS determination was hexa-deuterated clenbuterol (clenbuterol-D6) supplied by RIVM (Bilthoven, The Netherlands). LiChrosolv methanol (Merck, Darmstadt, Germany) was used as solvent for preparation of standard solutions. The reagents used in sample preparation and for obtaining and purifying extracts of urine and liver were sodium acetate, monobasic potassium phosphate, potassium hydroxide, 32% (*w*/*w*) aqueous ammonia, glacial acetic acid, methanol, 37% (*w*/*w*) hydrochloric acid, β-glucuronidase (30 U/ml) plus arylsulfatase (20 U/ml), all of which were acquired from Merck.

Ethyl acetate for chromatography (J.T. Baker, Deventer, The Netherlands) was used for elution of the analyte. Solid-phase extraction (SPE) columns containing 500 mg and 1000 mg (CleanScreen DAU, Bristol, USA) were used for urine and liver, respectively. The reagents for sample derivatization were MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) from Macherey–Nagel (Hoerdt, Germany), TMIS (trimethyliodosilane) from Sigma, and dithioerythritol from Merck.

Equipment

Beta-agonist-free bovine urine was homogenized on a magnetic mixer (IKA Mag-Ret, Wilmington, USA). A mixer cup (Kenwood A902, Havant, UK) was used for the grinding and homogenization of beta-agonist-free liver. For urine separation in 10.0 ml aliquots, a repetitive dispenser of 10.0 ml ± 0.02 ml (Optifix, Main, DE) was used. Fortified urine and liver were homogenized with a Reax 2000 vortex (Heidolph, Germany). Samples are stored at −20 °C ± 3 °C in a freezer (Hausetec H410.144.0, Rome, Italy); the temperature was monitored daily by means of a Dr Friedrichs Max–Min thermometer (Berlin, Germany). Storage at −60 °C ± 10 °C was done in an ultra-freezer fitted with a permanent thermal record of temperature (Revco, Asheville, USA). An ultracentrifuge (Beckman J2-21M/E, Glenrothes, UK) was used for centrifugation and SPE was undertaken with a vacuum system (J.T. Baker SPE, Philipsburg, USA). Extracts were evaporated to dryness with a Zymark (Hopkinton, USA) TurboVap-LV sample-concentration system under a current of compressed and dehydrated air supplied by a compressed air pump with an air dehumidification system (Alpro CS2000E, Germany). All pH adjustments were monitored with a pH meter equipped with a combined glass electrode coupled to a temperature probe (Metrohm, Herisau, Switzerland). A heating block (Grants QBT2, Cambridge, UK) and a Hewlett–Packard HP6890-HP5973 GC-MS system acquired from Soquimica (Lisbon, Portugal) with a HP-5MS chromatographic column (30.0 m × 0.25 mm ID × 0.25 μm film thickness) were also used.

Origin of beta-agonist-free biological samples

The beta-agonist-free matrices used were samples of bovine urine and liver analysed under the Azores Residue Control Plan, provided by the Regional Veterinary Laboratory of the Autonomous Region of the Azores (LRV) and confirmed as free from clenbuterol by the Portuguese Laboratory of Veterinary Investigation (LNIV), the National Reference Laboratory for this group of residues with CCα of 0.21 ng/ml and 0.45 ng/g, respectively for urine and liver.

Standard solutions

Two 1000.0 ng/ml clenbuterol (work standard—WS) and clenbuterol-D6 (internal work standard—IWS) solutions in

methanol were prepared. These solutions were kept at −20 °C during the research period.

Preparation of beta-agonist-free bovine urine aliquots and aliquots of bovine urine fortified with clenbuterol

The beta-agonist-free urine, frozen at −20 °C, was thawed, centrifuged (5000*g*, 20 min) and separated into aliquots of 10.0 ml. The aliquots needed for calibration curves were stored at −20 °C. Another fifty-four of these aliquots were fortified with 100.0 µl WS. Fifty-four 10.0-ml aliquots spiked with 10.0 ng/ml clenbuterol (UF aliquots) were thus obtained, of which six aliquots (∼11% of the batch) were separated at random (aliquot numbers 6, 13, 24, 35, 47, 54) and analysed on the same day (batch homogeneity study). Twenty-four aliquots (aliquot numbers 1, 3, 5, 8, 10, 12, 15, 17, 19, 21, 23, 26, 28, 30, 32, 34, 37, 39, 41, 43, 45, 48, 50, 52) were stored at −20 °C. Twenty-four aliquots (aliquot numbers 2, 4, 7, 9, 11, 14, 16, 18, 20, 22, 25, 27, 29, 31, 33, 36, 38, 40, 42, 44, 46, 49, 51, 53) were stored at −60 °C.

Preparation of beta-agonist-free bovine liver aliquots and aliquots of bovine liver fortified with clenbuterol

The beta-agonist-free liver stored at −20 °C was thawed, ground, homogenized, and separated into aliquots of 10.0 g. The aliquots needed for the calibration curves were stored at −20 °C with no addition of clenbuterol. An additional fifty-four aliquots were spiked with 100.0 µl WS. Fifty-four 10.0-g aliquots of liver fortified with 10.0 ng/g clenbuterol (FF aliquots) were thus obtained, of which six aliquots (∼11% of the batch) were separated at random (aliquot numbers 7, 14, 25, 36, 46, 53) and analysed on the same day (batch homogeneity study). Twenty-four aliquots (aliquot numbers 1, 3, 5, 8, 10, 12, 15, 17, 19, 21, 23, 26, 28, 30, 32, 34, 37, 39, 41, 43, 45, 48, 50, 52) were stored at −20 °C; and 24 aliquots (aliquot numbers 2, 4, 6, 9, 11, 13, 16, 18, 20, 22, 24, 27, 29, 31, 33, 35, 38, 40, 42, 44, 47, 49, 51, 54) were stored at −60 °C.

Stability tests

For the stability studies, in addition to the samples analysed on the first day to study homogeneity, aliquots of each batch stored during 4, 8, 12, 16, and 20 weeks at each temperature were analysed in triplicate.

Analytical methodology

The analytical methodology used for determination of clenbuterol in urine and liver was described previously [9, 13]. A brief summary is given below.

Obtaining urine extracts

IWS (100.0 µl) was added to a 10.0-ml aliquot of urine to obtain 10.0 ng/ml IS clenbuterol-D6. The pH was adjusted to 5.2 ± 0.3 with glacial acetic acid and then 4.0 ml acetate buffer, pH 5.2 ± 0.3, was added. The mixture was incubated with 50.0 µl *β*-glucuronidase plus arylsulfatase at 45 °C overnight. After cooling, 5.0 ml phosphate buffer, pH 6.0 ± 0.3, was added and the pH was readjusted to 6.0 ± 0.3 with 1.0 mol/l potassium hydroxide. The whole sample was then centrifuged (5500*g*, 5 min, 10 °C) and decanted.

The analyte was purified on an SPE column previously conditioned with 2.0 ml methanol, 2.0 ml water, and 2.0 ml phosphate buffer, pH 6.0 ± 0.3. The column containing the retained analyte was then impregnated with 1.0 ml 1 mol/l acetic acid (10.0 min), submitted to a vacuum (−800 mbar/10 min), washed with 6.0 ml methanol, again submitted to a vacuum (−800 mbar/10 min), and then eluted with 6.0 ml 97:3 (*v*/*v*) ethyl acetate–aqueous ammonia. The extract was evaporated to dryness at 45 °C under a current of dehydrated air [14].

Obtaining liver extracts

IWS (100.0 µl) was added to each 10.0-g aliquot of liver to obtain 10.0 ng/g IS clenbuterol-D6. The sample was ground and extracted in an Ultra-Turrax with addition of 20.0 ml acetate buffer, pH 5.2 ± 0.3. The mixture was incubated with 50.0 µl *β*-glucuronidase plus arylsulfatase at 45 °C overnight. After cooling, the sample was homogenized with hydrochloric acid (0.01 mol/l, 50.0 ml) and the pH was readjusted to 6.0 ± 0.3. The sample was then centrifuged (5000*g*, 30 min, 10 °C), decanted, and the supernatant was filtered.

The analyte was purified on an SPE column previously conditioned with 4.0 ml methanol, 4.0 ml water, and 4.0 ml phosphate buffer, pH 6.0 ± 0.3. The column containing the retained analyte was then impregnated with 2.0 ml 1.0 mol/l acetic acid (30 min), submitted to a vacuum (−800 mbar/10 min), washed with 12.0 ml methanol, again submitted to a vacuum (−800 mbar/10 min), and then eluted with 12.0 ml 97:3 (*v*/*v*) ethyl acetate–aqueous ammonia. The extract was evaporated to dryness at 45 °C under a current of dehydrated air.

Derivatization of urine and liver extracts

The dry extracts of urine and liver were derivatized with 50.0 µl MSTFA–TMSI–dithioerythritol, 1000:2:2 (*v*/*v*/*w*) at 60 °C ± 3 °C for 20 min [15].

GC–MS

The derivatized extracts were injected in splitless mode for 1 min; the injection volume was 2.0 µl and the injector temperature 270 °C. The carrier gas was helium, flow rate 1.4 ml/min. The chromatographic oven temperature was maintained at 100 °C for 0.4 min then programmed at 10°/min to 200 °C, which was maintained for 3 min, then programmed at 10°/min to 300 °C which was maintained 5 min.

The interface temperature was 280 °C and compounds were detected in electron-impact (EI) mode, with an electron energy of 70 eV. Data were acquired by selective ion monitoring (SIM) with ions of *m/z* 86, 300, 335 and 405 used for detection and identification of clenbuterol as the trimethylsilyl derivative. For quantitative determination ions of *m/z* 86 and 92 were used for clenbuterol and clenbuterol-D6, respectively.

Results and discussion

Results from study of the homogeneity of the urine and liver batches spiked with 10.0 ng/ml and 10.0 ng/g of clenbuterol, respectively, indicate that each batch can be considered homogeneous. Clenbuterol coefficients of variation (CV) in spiked urine aliquots (2.3%) and liver aliquots (3.7%) are not only very low but also much lower than the admissible CV for intralaboratory repeatability (between 16.0 and 21.0%, estimated through Horwitz's equation, for this concentration, as set down by Commission Decision (EC) 657/2002 [16]).

Data from studies of the stability of clenbuterol in spiked urine and liver stored at either −20 °C and −60 °C are presented in Fig. 1. Results from linear regression analysis for each kind of sample at each one of the temperatures studied, the mean concentration of clenbuterol, and the respective coefficients of variation obtained during the studies can be seen in Table 1.

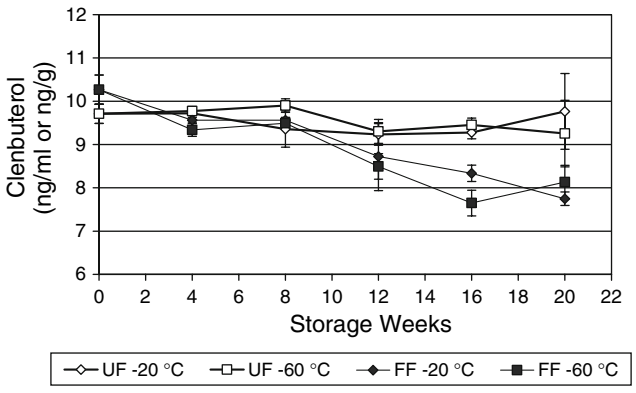

Fig. 1 Results from study of the stability of clenbuterol in bovine urine fortified with 10 ng/ml clenbuterol (UF) and bovine liver fortified with 10 ng/g clenbuterol (FF) and stored at −20 °C and −60 °C (sixfold determination on first day and threefold determination on each of the subsequent five days, at each temperature)

For spiked urine the results show that the bias found for the aliquots stored at either −20 °C and −60 °C for 20 weeks does not differ significantly from zero ($P > 0.05$), which means that clenbuterol can be regarded as stable in this matrix. The low CV for clenbuterol concentration during storage at −20 °C (3.2%) and at −60 °C (3.9%) corroborate this conclusion, because the CV obtained are rather low and are indeed lower than the coefficient of variation of the method itself, as estimated from the daily calibration curves (6.2%, $n = 30$, in the concentration range 0.0 to 16.0 ng/ml). These data do not agree with those from a study undertaken by Gigosos et al. [17], in which a decrease of clenbuterol concentration was observed in bovine urine fortified with 10.0 ng/ml and stored at −15 °C for 6 months. It is thus possible to consider the hypothesis that the storage temperature may have influenced clenbuterol recovery.

Results of linear regression analysis of clenbuterol concentration in spiked liver during stability studies show that the bias found for the concentration of clenbuterol of the stored aliquots, at both −20 °C and −60 °C, for 20 weeks, differs very significantly from zero for both temperatures ($P < 0.05$). These results demonstrate that aliquots of bovine liver homogenized and spiked with 10.0 ng/g clenbuterol are unstable when stored for 20 weeks at either of these temperatures. More to the point, at −20 °C, both a decrease in clenbuterol concentration of about 0.1 ng/g of liver per week and a total decrease of 2.4 ng/g after the 20 weeks of storage were observed. At −60 °C a decrease in clenbuterol concentration of about 0.1 ng/g of liver per week and a total decrease of 2.4 ng/g after the 20-weeks of storage were also observed. The CV of clenbuterol concentrations from the beginning to the end of the experiment obtained at both −20 °C and −60 °C are 10.5 and 11.1%, respectively. These CV values are higher than the coefficient of variation of the method estimated from the calibration curves obtained in intralaboratory reproducibility conditions (7.7%, $n = 30$, in the concentration range 0.0 to 16.0 ng/g), which also highlights the significant lack of stability observed in this study.

This study enables consideration of the hypothesis, defended by Gude et al. [18], that liver homogenization activates enzymes which lead to clenbuterol degradation, and that this degradation takes place even when the liver is stored frozen, not only at the temperature studied by Gude et al. (−30 °C), but also at −20 °C and −60 °C. Clenbuterol degradation may have occurred, however, not just by activation of enzymes during the grinding procedures prior to sample freezing but also as a result of use of

Table 1 Results from study of the stability of clenbuterol in bovine urine fortified with 10 ng/ml clenbuterol (UF) and in bovine liver fortified with 10 ng/g clenbuterol (FF)

Sample	Storage temperature(°C)	Slope	Standard error of slope	Significance (p value)	Mean clenbuterol (ng/ml)	CV (%)	Storage (weeks)
UF	−20	−0.009	0.016	0.621	9.54 ($n = 21$)	4.30	20
	−60	−0.027	0.011	0.073	9.59 ($n = 21$)	3.92	20
FF	−20	−0.122	0.011	0.000(4)	9.21 ($n = 21$)	10.54	20
	−60	−0.120	0.026	0.009	9.10 ($n = 21$)	11.09	20

Simple linear regression analysis was performed, with a Student t test for slope ($N - 2$ degrees of freedom and 95% confidence, $N = 6$-days of analysis). Mean concentration of clenbuterol and the respective coefficient of variation (CV) are given for each storage temperature and each type of sample for all storage times

the soluble free form (clenbuterol.HCl) with which the aliquots were spiked. However, the main source of instability could arise because the water content of the liver was not reduced to an optimum level with respect to water activity. This is especially relevant for microbiological degradation but is also important for degradation because of enzymatic activity, oxidation, and condensation reactions. Taking several studies into consideration, Linsinger et al. [19] have concluded that water activities between 0.15 and 0.30 appear to ensure the minimum possible degradation rate.

As for the possibility of producing "in house" reference materials consisting of urine or liver spiked with clenbuterol in its soluble form (clenbuterol.HCl), this study leads to different conclusions. The study concludes that such a possibility exists for urine, because significant stability ($P > 0.05$) of clenbuterol was demonstrated in urine fortified with 10.0 ng/ml and stored at either −20 °C or −60 °C for 20 weeks. In this situation it is possible to estimate the uncertainty of clenbuterol measurement in urine at the spiked level, for both temperatures, according to Linsinger [19]. For the batch of spiked urine stored at −20 °C, for example, the concentration of clenbuterol (9.54 ng/ml) has an associated uncertainty of 4.30% (or an uncertainty of 8.6% after multiplication by a coverage factor of two for a confidence level of approximately 95%, equivalent to an uncertainty of 0.82 ng/ml).

In contrast, for homogenized liver spiked with clenbuterol at 10.0 ng/g it was demonstrated that clenbuterol is significantly unstable ($P < 0.05$) at both −20 °C and −60 °C during a 20-week storage period. This instability demonstrates that it is not possible to produce "in house" reference materials with the matrix liver under the conditions studied. The production of such materials under these conditions will only be possible at the cost of greater uncertainty.

Therefore there remains a need for reference materials comprising bovine liver containing just a single beta-agonist, namely clenbuterol, for validation of screening methods, particularly for the validation of ELISA-type tests, for which recovery and cross reactions are extremely variable. Since these materials are considered indispensable for reliability in validation procedures, the official entities producing certified reference materials, for example the Institute for Reference Materials and Measurements (IRMM) or the National Institute for Public Health and the Environment (RIVM), are challenged to promote their production in the form of positive and lyophilized liver, the stability of which has already been demonstrated. The future production of certified reference materials containing only clenbuterol, in matrices of interest, will by an extremely useful tool for validation of the ELISA-type screening methods that are already widely used in routine laboratories.

Acknowledgments The authors are grateful to the Portuguese Foundation for Science and Technology (POCTI-SFA-8-177), to the Calouste Gulbenkian Foundation, and to the Services of the Secretary of Agriculture and Forestry (SRAF) of the Regional Government of the Autonomous Region of the Azores, for financial support of this study.

References

1. Council Directive 96/23/EC (1996) Off J Eur Uni L 125:10–32
2. Directive 2003/74/CE (2003) Off J Eur Uni L 262:17–21
3. Martinez-Navarro JF (1990) Lancet 336:1311
4. Garay BJ, Jiménez JFH, Jiménez ML, Sebastian MV, Matesanz JP, Moreno PM, Galiana J (1997) Rev Clin Esp 197(2):92–95
5. Pulce C, Lamaison D, Keck G, Bostvironnois C, Nicolas J, Descotes J (1991) Vet Hum Toxicol 33(5):480–481
6. Maistro S, Chiesa E, Angeletti R, Brambilla G, (1995) Lancet 346:180
7. Sporano V, Grasso L, Esposito M, Oliviero G, Brambilla G, Loizzo A (1998) Vet Hum Toxicol 40(3):141–143
8. Brambilla G, Cenci T, Franconi F, Galarini R, Macri A, Rondoni F, Strozzi M, Loizzo A (2000) Toxicol Lett 114(1–3):47–53
9. Barbosa J, Cruz C, Martins J, Silva JM, Neves C, Alves C, Ramos F, Silveira MIN (2005) Food Addit Contam 22(6):563–566
10. Shiu TC, Chong WH (2001) Publ Health Epidemiol Bull 10:14–17
11. Cunningham NF, Heitzman RJ (2001) European Commission EUR 19452/EN, bcr-503

12. Gaspar P, van-Vyncht G, Scippo ML, Maghuin-Rogister G, Kramer GN, Gawlik BM, Linsinger T, Lamberty A, van-Ginkel LA, Dirscherl C (2002) ISBN 92-894-4657-9-EUR 20526/EN, bcr-649
13. Montrade MP, le Bizec B, Monteau F, Siliart B, André F (1993) Anal Chim Acta 275:253–268
14. Ramos F, Bañobre MC, Castilho MC, Silveira MIN (1999) J Liq Chromatogr Related Technol 22:2307–2320
15. Cristino A, Ramos F, Silveira MIN (2003) J Pharm Biomed Anal 32:311–316
16. Commission Decision 2002/657/EC (2002) Off J Eur Uni L221:8–36
17. Gigosos PG, Sampayo CF, Abuín CF, Sáez AC, Belda BIV (2002) Food Addit Contam 19(11):1010–1014
18. Gude T, Schmädicke I, Rahn S, Benesch-Girke L, Hashem A, PreißA, Jülicher B (1996) In: Haagsma N, Ruiter A (eds) Residues of veterinary drugs in food: Proceedings of the EuroResidue III conference, pp 157–161
19. Linsinger TPJ, Pauwels J. Van der Veen AMH, Schimmel H, Lamberty A (2001) Accred Qual Assur 6:20–25